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DATA EVALUATION REPORT

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STUDY TYPE:	Food Allergenicity-Brown Norway Rat Model
MRID NO:	447140-02
CHEMICAL NO:	006466
TEST MATERIAL:	Cry9C protein from *Bacillus thuringiensis* ssp. *tolworthi*
STUDY NO:	Contract No. EU AIR3 CT94-2311
SPONSOR:	AgrEvo USA Company, Wilmington, DE
TESTING FACILITY:	BIBRA International, Carshalton, Surrey, UK
TITLE OF REPORT:	Development of New Methods for Safety Evaluation of Transgenic Food Crops
AUTHOR:	Sally Van Wert, Ph.D.; Dr. Clive Meredith
STUDY COMPLETED:	20 November 1998
CONCLUSION:	The results of intraperitoneal injection of corn powder extracts into BN rats indicate that both the control and transgenic corn powders are able to induce IgE or reagininc antibody responses by the PCA assay. The use of corn powder immunogen decreases the rate of the immune response to the Cry9C protein compared to the bacterial preparation. However, the lowest responding dose for Cry9C was similar for the two preparations (between 0.1 and 0.4 µg Cry9C). The control challenge test with the heterologous antigen of control corn powder or transgenic corn powder in the day 42 sera samples indicate that there was significant reactivity form the corn portion of the extracts themselves in the PCA assay. It is unclear, given this background reactivity, how suppositions can be made about the reactivity of the Cry9C protein alone. The PCA results from oral sensitization with ovalbumin II, control corn extract, bacterial Cry9C and transgenic corn (apparently supplemented with bacterial Cry9C) indicated that an IgE or reagin antibody response was elicited in naïve Sprague-Dawley rats. Ovalbumin produced a low frequency of responders and a weak dose response between the 5.0 and 50.0 mg/kg dose levels on days 28 through 42. The control corn also produced a positive oral sensization response, but this was only examined at the 50mg/kg dose. Oral dosing with bacterial Cry9C gave a positive PCA response as did the Cry9C amended transgenic corn extract. The frequency of response to bacterial Cry9C began to diminish in day 42 sera. The Cry9C amended transgenic corn had a higher frequency of responders and the frequency remained high on day 42 PCA response. Western blot analysis indicated that Cry9C protein bands could be recognized in the rat sera from both exposure routes.
CLASSIFICATION:	Supplemental. A more detailed description of the materials and methods, especially how the PCA system was utilized in these experiments, is needed. Specifically: 1) how long after the serum

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injections were the challenge antigen injections done; 2) what was the concentration of Cry9C in the bacterial and corn plant powder preparations; 3) The source of the test animals and their care during the test were not described; 4) How many naïve Sprague-Dawley rats were used for each serum tested; 5) What were the individual animal responses; 6) The original gel for the western blot assay should be provided.

STUDY DESIGN

The brown Norway (BN) rat model for the detection of food allergens has been used to determine the potential of the Cry9C protein to induce a food sensitization response. The model has not been validated and the study was not conducted according to the 40 CFR 160 GLP standards. In addition there were results from an initial antibody production study to develop a western blot detection system and a sensitization assay with both Cry1Ab5 and Cry9C proteins reported but not described.

Test material: Bacterially produced Cry1Ab5 and Cry9C were provided by Plant Genetic Systems, Ghent, Belgium. There were four batches of the bacterial produced Cry9C protein extracts provided at the following amounts: 1) 20mg of a white powder; 2) 1.6g of a buff powder; 3) 60 mg of a white powder and 4) 10g of a buff powder. There was no analysis of these bacterial powders for purity or Cry9C content. Samples of corn powder extract from both non-transgenic plants and plants expressing the Cry9C protein were also provided by PGS. These extracts are called "whole corn plant powder." There were also two batches of corn powder provided: 1) the transgenic Cry9C corn powder with 4% Cry9C (TPPE-351-0197) and the non-transgenic corn powder (TPPE-351-0197c) free of Cry9C; 2) the transgenic corn powder with 4.7% and the non-transgenic corn with 0.05% detectable Cry9C. There was no indication that the testing lab did these concentration studies. Ovalbumin grade II was obtained from Sigma Chemical Company Ltd., Poole, Dorset, U.K. The solutions used in these experiments were prepared by dispersing them in phosphate buffered saline, mixing, homogenizing and separating the aqueous fraction by centrifugation.

Test animals: Groups of ten brown Norway (BN) rats were used for the initial study with intraperitoneal injection for the sensitization phase. Groups of eight BN rats were used for the oral sensitization phase. Naïve Sprague-Dawley rats were used for the passive cutaneous anaphylaxis (PCA) tests with sera obtained from the BN rats. No details were given as to where the animals used in these studies were obtained, their general health nor how they were maintained during the test.

Test methods:

Passive Cutaneous Anaphylaxis: The basis of these studies is the PCA assay where serum from a sensitized animal is subcutaneously injected into a naïve animal. Any IgE (also termed reagin) present in the injected serum will bind mast cells present in the naïve animal. Subsequently, the alleged allergen together with a protein binding dye is intravenously injected into the tail vein of the naïve animals. If a mast cell degranulation reaction occurs, the area around the subdermal injection site will become infiltrated with dye as the blood vessels near the site become more permeable due to the histamine and other modulators released during degranulation. The intensity of the reaction is expressed as the area of dye infiltrated skin near the subcutaneous injection site. This

hypersensitivity phenomenon was originally described in 1921 as is sometimes referred to as the Prausnitz and Kustner reaction.

Sensitization by intraperitoneal injection: The initial study was performed with groups of ten BN rats injected with 1.0, 10 or 100µg of transgenic or non-transgenic corn powder. The extract was mixed with 10 mg of carrageenan in 1 ml of saline. Carrageenan is reported to be a good adjuvant for the IgE response in BN rats. The amount of Cry9C protein in these extracts was 0.04, 0.4 or 4.0 µg. It is unclear from the description whether there was more than one injection prior to the PCA assays. Blood samples were taken from the treated BN rats at 14, 21, 28, 35 and 42 days. These serum samples we tested for IgE response with the PCA assay using naïve Sprague-Dawley rats. Cross-reactivity was tested with sera taken on day 42 as follows: serum from the Cry9C corn powder sensitized individuals was tested against a challenge with non-transgenic corn powder and the non-transgenic corn powder sensitized individuals were challenged with transgenic corn powder.

Sensitization by the oral route: This study was done by exposing groups of eight BN rats to four different antigen treatments: 1) bacterial Cry9C (batch 4); 2) ovalbumin II; 3) control corn powder (TPPE-351-0197c, batch 2) or 4) transgenic corn powder (TPPE-351-0197, batch 2). The dosing was done with 0.5ml/100g bodyweight twice a week for six weeks. No description of the composition of the dosing solutions was given nor could it be determined if dosing was done by gavage or drinking water. However, data tables indicate a dose range of 1.0, 2.0 and 10.0mg/ml or 5, 10 and 50mg/kg bodyweight for the ovalbumin II and Cry9C by oral sensitization. The BN rats were also given intraperitoneal injections of carrageenan (1mg in 1ml of saline) once a week for six weeks prior to the oral sensitization phase. Blood samples were taken from the rats at weekly intervals after the sensitization (days 14, 21, 28, 35 and 42). The PCA tests were done with naïve Sprague-Dawley rats which included as controls challenges with non-transgenic corn or Cry9C amended corn extracts.

RESULTS AND DISCUSSION

The reported results of the studies are attached as an appendix to this review. The experimental method for some of the data reported in the tables were not described in the materials and methods sections. Tables 1 & 2 indicate that both Cry1Ab5 and Cry9C are capable of inducing an IgE or reagininc antibody response in BN rats following intraperitoneal injection with bacterial preparations by the PCA assay. The authors claim that Cry9C has a lower response threshold since a higher frequency of responding BN rats were observed than for Cry1Ab5.

In tables 3 & 4, the results of intraperitoneal injection of corn powder extracts into BN rats indicate that both the control and transgenic corn powders are able to induce IgE or reagininc antibody responses by the PCA assay. The authors again claim that the response for Cry9C was greater due to the higher number of responders and the earlier onset of IgE response.

Comparing the serum response between tables 2 & 4 indicates the use of corn powder immunogen decreases the rate of the immune response to the Cry9C protein compared to the bacterial preparation (day 21 compared to day 14). However, the lowest responding dose for Cry9C was similar for the two preparations (between 0.1 and 0.4 µg Cry9C). The control challenge test with the heterologus antigen of control corn powder or transgenic corn powder in the day 42 sera samples indicate that

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there was significant reactivity form the corn portion of the extracts themselves in the PCA assay. It is unclear, given this background reactivity, how suppositions can be made about the reactivity of the Cry9C protein alone.

In table 5, sera from the control corn powder intraperitoneal injection sensitized animals did not react with bacterial Cry9C. The response of transgenic corn sensitized serum to non-transgenic corn, transgenic corn or bacterial Cry9C extracts was similar. The response of bacterial Cry9C sensitized sera to different batches of bacterial Cry9C were positive and it appeared that there were higher concentrations of the Cry9C protein present in batches 2 and 3.

In tables 6 & 7, the PCA results from oral sensitization with ovalbumin II, control corn extract, bacterial Cry9C and transgenic corn (apparently supplemented with bacterial Cry9C) indicated that oral exposure elicited an IgE or reagin antibody response in the naïve Sprague-Dawley rats. The ovalbumin sensitized serum had a low frequency of responders and showed a weak dose response between the 5.0 and 50.0 mg/kg dose levels on days 28 through 42. The control corn also gave a positive oral sensization response but this was examined only at a single dose (50mg/kg). Oral dosing with bacterial Cry9C gave a positive PCA response as did the Cry9C amended transgenic corn extract. The frequency of response to bacterial Cry9C began to diminish in day 42 sera. The Cry9C amended transgenic corn produced a higher frequency of responders, which remained high on day 42.

Finally, a western blot analysis of sensitized rat sera from both the oral and intraperitoneal exposure methods was presented (figure 4). Reactive bands were observed at several locations including the 66kDa band expected for Cry9C, another band at approximately 100kda and also several lower molecular weight bands. There are also positive bands at these same regions in the control non-transgenic corn extracts. One of the problems with interpreting this figure is that it has been highly manipulated by the digitized presentation format. The Coomassie blue stained lanes are very weakly stained which is unlikely in a plant extract with any significant protein content. A matchup between the low and the high molecular weight markers on either end of the gel is not possible. Overall, the bands, while separated in the presentation format used, indicate that there was probably a smile in the banding pattern which is extremely difficult to evaluate if the original gel is not presented.

REVIEWER COMMENT

It is unclear from the studies or the literature provided that the idea of "relative potency" for proteins to be food allergens is a valid concept. That a protein can be shown to induce an IgE response is a first step towards identifying food allergen potential. The fact that many humans are IgE serum positive for a protein that does not correlate with an expressed allergy in these people would confound that assumption. The detection of mast cell degranulation by the PCA system would be a next step. However, it is not apparent from the provided materials and methods description that appropriate measures were taken to verify the PCA test. According to the cited literature, the antigen/dye injection was done 24 hours after subcutaneous serum injection. This is less than the normal 48-72 hours required to allow diffusion of cross reacting IgG which could also cause mast cell degranulation and give a false positive reaction. Another proof against false positive reactions would be the heat treatment of test serum to inactivate IgE and confirm that the response was due to antigen specific IgE. It would have been clearer to include proteins that have not been implicated

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as food allergens (i.e., bovine serum albumin) to test the model for false positives. According to other information included in the data package, standard protocol in the PCA system is to dilute out the test sera to determine the level of serum IgE reactivity, not to note the frequency of responders and area of the dye extravasation.

An independent determination of the concentration of the Cry9C protein in the bacterial preparations tested is essential. The oral sensitization schedule is unclear as to when the carrageenan intraperitoneal adjuvant was stopped and when oral dosing with the various antigens began. The western blot data presented needs a materials and methods section to describe the process and sera used and inclusion of the original gel or membrane in order to render any conclusions about serum reactivity.

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Appendix to BN RAT STUDY

Table 1. Reaginic antibody response in those animals developing a response following exposure to Cry1ab5 by the intraperitoneal route.

Day		Area of dye extravasation mm²				
		Cry1ab5 μg/ml				
		100	10	1.0	0.1	0.01
14	Mean	302	313	242	0	0
	SD	85.0	116.0	91.3	0.0	0.0
	Responders	7/8	4/8	4/8	0/8	0/8
21	Mean	307	225	245	0	0
	SD	92.2	109.1	66.4	0.0	0.0
	Responders	7/8	4/8	4/8	0/8	0/8
28	Mean	314	229	197	0	0
	SD	88.3	49.0	28.5	0.0	0.0
	Responders	7/8	4/8	4/8	0/8	0/8
35	Mean	302	210	189	0	0
	SD	91.2	54.1	13.9	0.0	0.0
	Responders	7/8	4/8	4/8	0/8	0/8
42	Mean	260	222	195	0	0
	SD	77.9	46.0	12.0	0.0	0.0
	Responders	7/8	4/8	4/8	0/8	0/8

Table 2. Reaginic antibody response in those animals developing a response following exposure to Cry9c by the intraperitoneal route.

Day		Area of dye extravasation mm²				
		Cry9c μg/ml				
		100	10	1.0	0.1	0.01
14	Mean	214	319	253	217	0
	SD	82.7	82.3	30.6	128.5	0.0
	Responders	4/8	7/8	8/8	6/8	0/8
21	Mean	249	297	290	206	0
	SD	66.5	53.3	60.0	94.6	0.0
	Responders	6/8	7/8	8/8	6/8	0/8
28	Mean	216	260	264	232	0
	SD	22.4	68.7	77.9	103.8	0.0
	Responders	6/8	7/8	8/8	6/8	0/8
35	Mean	128	126	229	161	0
	SD	25.2	32.0	102.0	24.6	0.0
	Responders	6/8	7/8	8/8	6/8	0/8
42	Mean	138	201	245	182	0
	SD	50.9	80.3	114.0	120.0	0.0
	Responders	6/8	7/8	8/8	6/8	0/8

Table 3. Reaginic antibody responses in those animals developing a response following exposure to Control corn powder extract by the intraperitoneal route.

Control corn powder extract μg		Area of dye extravasation mm²					
		Day					
		14	21	28	35	42	42*
1	Mean	0	0	0	333	209	284
	SD	0.0	0.0	0.0	0.0	106.8	0.0
	Responders	0/10	0/10	0/9	1/9	2/10	2/8
10	Mean	0	0	104	128	174	225
	SD	0.0	0.0	80.4	140.7	136.4	68.1
	Responders	0/10	0/10	3/10	2/10	4/10	4/10
100	Mean	0	28	127	151	127	167
	SD	0.0	0.0	29.9	140.7	105.8	50.7
	Responders	0/10	1/10	3/10	3/10	4/10	6/10

PCA challenge carried out using control corn powder extract, * PCA challenge carried out using transgenic corn powder extract

Table 4. Reaginic antibody responses in those animals developing a response following exposure to transgenic corn powder exract by the intraperitoneal route.

Transgenic corn powder extract μg (Cry9C μg)		Area of dye extravasation mm²					
		Day					
		14	21	28	35	42	42*
1 (0.04)	Mean	0	0	0	0	0	0
	SD	0.0	0.0	0.0	0.0	0.0	0.0
	Responders	0/10	0/10	0/10	0/10	0/10	0/10
10 (0.4)	Mean	0	150	136	180	138	145
	SD	0.0	69.9	74.4	47.4	86.3	45.3
	Responders	0/10	6/10	5/10	6/10	6/10	2/10
100 (4.0)	Mean	0	195	257	164	206	125
	SD	0.0	67.8	76.4	31.6	27.7	92.6
	Responders	0/10	8/10	8/10	8/10	7/10	6/10

PCA challenge carried out using bacterial Cry9C, * PCA challenge carried out using control corn powder extract

Table 5. Examination of specificity of PCA responses to Cry9C in sera obtained following exposure to different batches of bacterial Cry9C, control corn extract and transgenic corn extract by the intraperitoneal routes.

		ADE mm²		
Treatment		PCA Challenge material		
Control corn powder extract (1)		Control corn	Transgenic corn	Cry9C (3)
An.No	9.56	255	201	0
	9.61	64	33	0
	9.62	64	154	0
	9.65	95	227	0
		120	154	
		95	96	
Transgenic corn powder extract (1)		Control corn	Cry9C (3)	Cry9C (3)
An.No.	9.90	177	177	177
	9.91	177	113	201
	9.96	284	227	201
	9.98	85	201	177
		181	180	191
		81	92	13.9
Cry9c batch 1		Cry9C (1)	Cry9C (2)	Cry9C (3)
An.No.	7.32	201	531	573
	7.37	177	380	416
	7.40	177	346	346
		185	419	445
		13.9	98.5	116.2

Cry9C (total quantity received at BIBRA from PGS)

batch 1 20 mg
batch 2 1.6 g
batch 3 60 mg
batch 4 10 g

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Table 6. Reaginic antibody response in those animals developing a response following exposure to Ovalbumin II or control corn by the oral route.

		Area of dye extravasation mm²			
		Ovalbumin II			Control Corn *
Day	mg/ml (mg/Kg)	10.0 (50)	2.0 (10)	1.0 (5)	10.0 (50)
14	Mean	0	0	0	0
	SD	0.0	0.0	0.0	0.0
	Responders	0/8	0/8	0/8	0/8
21	Mean	0	0	0	33
	SD	0.0	0.0	0.0	0.0
	Responders	0/8	4/8	0/8	1/8
28	Mean	184	0	0	167
	SD	100.4	0.0	0.0	48.1
	Responders	2/8	0/8	0/8	2/8
35	Mean	347	197	177	195
	SD	46.7	111.2	0.0	148.7
	Responders	2/8	2/8	1/8	5/8
42	Mean	226	173	201	166
	SD	154.8	64.8	0.0	81.0
	Responders	4/8	3/8	1/8	7/8

* PCA challenge with control corn

Table 7. Reaginic antibody response in those animals developing a response following exposure to Cry9C or transgenic corn by the oral route.

		Area of dye extravasation mm²			
		Cry9C			Transgenic Corn : Cry9C **
Day	mg/ml (mg/Kg)	10.0 (50)	2.0 (10)	1.0 (5)	10.0 : 0.045 (50 : 0.225)
14	Mean	0	0	0	491
	SD	0.0	0.0	0.0	0.0
	Responders	0/8	0/8	0/8	1/8
21	Mean	124	227	0	222
	SD	91.8	0.0	0.0	63.8
	Responders	3/8	1/8	0/8	7/8
28	Mean	283	339	270	375
	SD	77.6	199.0	83.9	209.2
	Responders	6/8	6/8	6/8	7/8
35	Mean	230	382	331	287
	SD	145.2	234.9	146.6	108.1
	Responders	4/8	6/8	6/8	8/8
42	Mean	230	227	255	264
	SD	0.0	0.0	0.0	82.9
	Responders	1/8	1/8	1/8	7/8

** PCA Challenge with Cry9C

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Table 8. Examination of specificity of PCA responses to Cry9C in sera obtained following exposure to different batches of bacterial Cry9C, control corn extract and transgenic corn extract by the oral route.

		ADE mm^2	
Treatment		PCA challenge material	
Control corn powder extract (2)		Control corn	Cry9C (3)
An.No	11.49	177	312
	11.53	227	284
	11.54	201	255
	11.55	255	255

Transgenic corn powder extract (2)		Cry9C (4)	Cry9C (3)
An.No.	11.57	177	300
	11.58	227	255
	11.59	284	314
	11.60	314	416
	11.61	227	154
	11.62	419	255
	11.63	201	154
	11.64	0	0

Cry9C (total quantity received at BIBRA from PGS)

batch 1 20 mg
batch 2 1.6 g
batch 3 60 mg
batch 4 10 g

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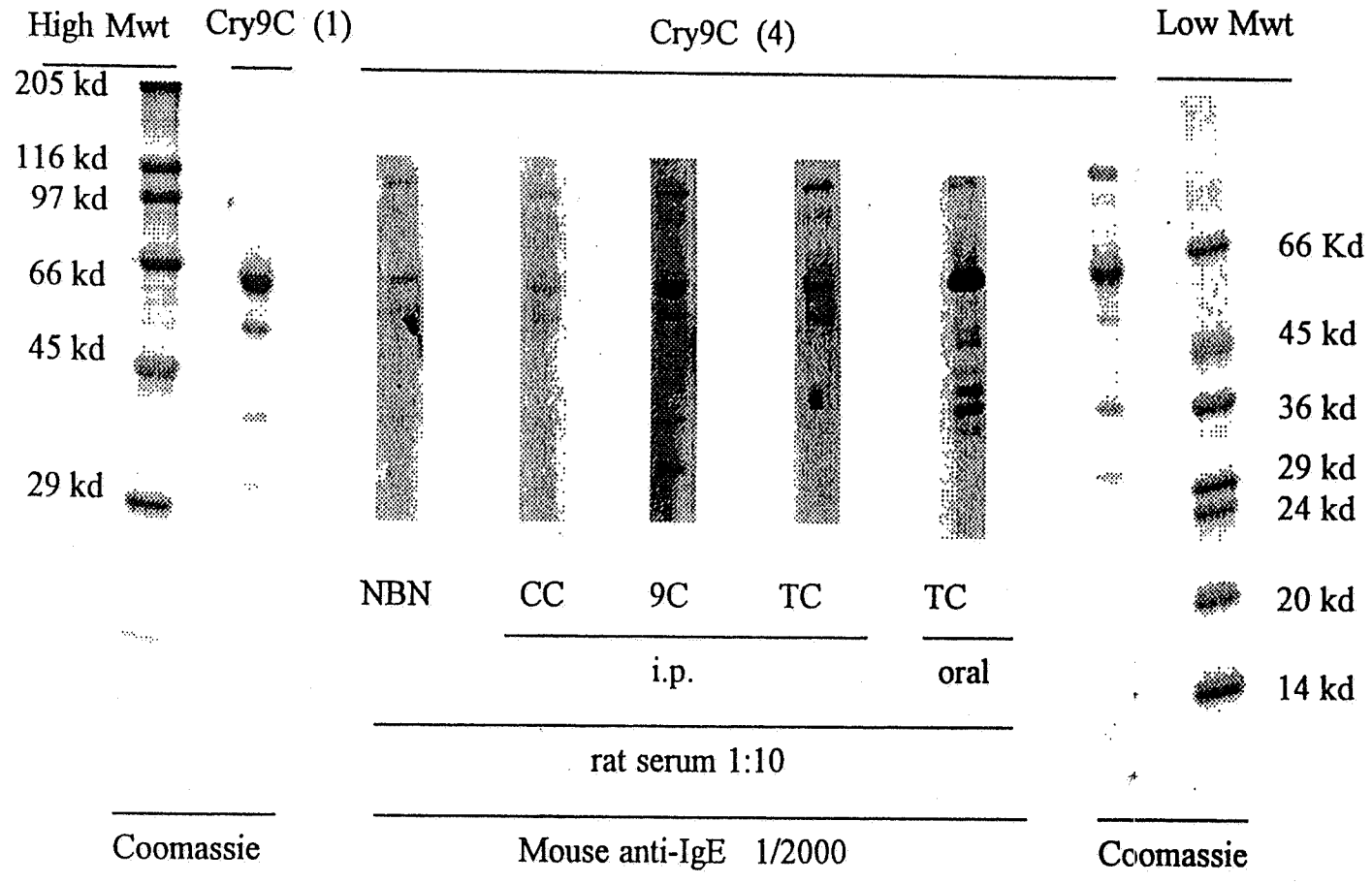
Figure 4.


High Mwt
Cry9C (1)
Cry9C (4)
Low Mwt
205 kd
116 kd
97 kd
66 kd
45 kd
29 kd
66 Kd
45 kd
36 kd
29 kd
24 kd
20 kd
14 kd
NBN
CC
9C
TC
TC
i.p.
oral
rat serum 1:10
Coomassie
Mouse anti-IgE 1/2000
Coomassie